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(71) Applicant *(for all designated States except US)*: **INEX PHARMACEUTICALS CORP.** [CA/CA]; 100 - 8900 Glen Lyon Parkway, Burnaby, British Columbia V5J 5J8 (CA).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **KNOPOV, Victor** [CA/CA]; 306 - 7560 Moffatt Road, Richmond, British Columbia V6Y 1X8 (CA). **DZUBANOV, Kirill** [CA/CA]; 120A - 904 Clarke Road, Port Moody, British Columbia V3H 1L5 (CA). **HARPER, Kevin** [CA/CA]; 12503 - 28th Avenue, Surrey, British Columbia V4A 2N8 (CA). **CULLIS, Pieter, R.** [CA/CA]; 3272 West 1st Avenue, Vancouver, British Columbia V6R 1H4 (CA).

(74) Agents: **KINGWELL, Brian, G.** et al.; Smart & Biggar, Vancouver Centre, Suite 2200, 650 West Georgia Street, Box 11560, Vancouver, British Columbia V6B 4N8 (CA).

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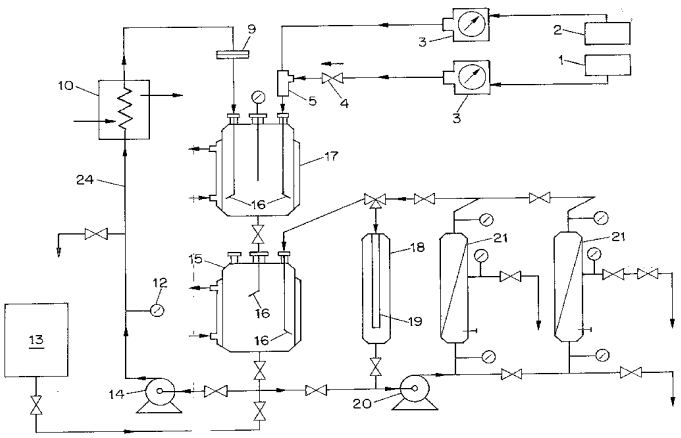
(54) Title: METHODS AND APPARATUS FOR PREPARATION OF LIPID VESICLES



(57) Abstract: Improved production of lipid vesicles is achieved using an apparatus for preparation of a lipid vesicle that includes: (a) a first reservoir for receiving a buffer composition; (b) a static mixer for agitating buffer composition in the first reservoir; (c) a second reservoir for receiving a lipid solution; (d) a dispensing head for introducing lipid solution from the second reservoir into the first reservoir, and (e) a connector joining the second reservoir to the dispensing head for conducting lipid solution from the second reservoir to the dispensing head. The dispensing head has formed therein one or more injection ports having a diameter of 2 mm or less. To use the apparatus, one first prepares a solution of ethanolic lipid comprising from about 1 to about 100 mg/ml lipid in at least 90% by weight ethanol. This ethanolic lipid is injected directly into aqueous buffer through the injection port to make a lipid/buffer mixture, which is mixed by turbulent passage through a static mixer. The resulting lipid vesicles, prior to an extrusion step, are in about 10 % or more by weight ethanol, and have average diameter of from about 80 nm to about 200 nm.

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Methods and Apparatus for Preparation of Lipid Vesicles

Field of the Invention

This invention relates to novel methods and apparatus for making lipid vesicles for use in the preparation of therapeutic agents.

Background of the Invention

Hydration is a critical step of liposome formation. Hydration occurs when lipids are transformed from dry crystals or non-aqueous solutions into aqueous or partially aqueous solution. Under certain conditions, hydration has the effect of forming enclosed liposomes. The type of hydration step influences the type of liposome formed (i.e. number of layers, size, and entrapped volume). Different types of hydration steps essentially distinguish the known liposome manufacturing techniques. Large scale manufacturing techniques for lipid particles can be broadly classified into the following categories: 1) Lipid Film Hydration (i.e. Passive entrapment); 2) Reverse Phase Evaporation; 3) High-Pressure extrusion; 4) and Solvent injection (dilution) (see for example US Patent Nos. 4752425 and 4737323 to Martin et al).

Particular instruments for lipid particle manufacturing disclosed in the art include: US Patent Nos. 5270053 and 5466468 to Schneider et al; Isele, U. et al. (1994) Large-Scale Production of Liposomes Containing Monomeric Zinc Phthalocyanine by Controlled Dilution of Organic Solvents. J. Pharma. Sci. vol 83(11) 1608-1616; Kriftner, RW. (1992) Liposome Production: The Ethanol Injection Technique, in Bruan-Falco et al., eds, Liposome Derivatives, Berlin, Springer -Verlag, 1992, pp. 91-100; Kremer et al. (1977) Vesicles of Variable Diameter Prepared by a Modified Injection Method. Biochemistry 16(17): 3932-3935; Batzri, S. and Korn, ED. (1973) Single Bilayer Liposomes Prepared Without Sonication, Bioch. Biophys. Acta 298: 1015-1019.

Commercial large scale manufacturing of liposomes is not efficiently achieved using existing methods and instruments. This problem remains notwithstanding that for two decades many investigators have attempted to understand the vesiculation/hydration process at large scale volumes. Problems include the wide size range of liposomes produced; large median diameters of particles that must be reduced for

therapeutic applications; heterogeniety of resulting compositions; and requirement for extensive post-hydration processing.

It is highly desirable to obtain an efficient, high volume methods and instruments for generating homogeneous liposomes that require a minimum of post-hydration processing. The instant invention provides, for the first time, methods and instruments for the generation of liposomes employing continuous flow hydration (including use of a static mixer) and requiring no post-hydration extrusion or size reduction step. The resulting liposomes may be used in therapeutic compositions and for experimentation and otherwise. It is an object of this invention to provide such methods and instruments.

SUMMARY OF THE INVENTION

In accordance with the invention, improved production of lipid vesicles is achieved using an apparatus for preparation of a lipid vesicles comprising:

(a) a first reservoir for receiving a buffer composition;

(b) a static mixer for agitating buffer composition in the first reservoir;

(c) a second reservoir for receiving a lipid solution;

(d) a dispensing head for introducing lipid solution from the second reservoir into the first reservoir, and

(e) a connector joining the second reservoir to the dispensing head for conducting lipid solution from the second reservoir to the dispensing head. The dispensing head has formed therein one or more injection ports having a diameter of 2 mm or less.

To use the apparatus, one first prepares a solution of ethanolic lipid comprising from about 1 to about 100 mg/ml lipid in at least 90% by weight ethanol. This ethanolic lipid is injected directly into aqueous buffer through the injection port to make a lipid/buffer mixture, which is mixed by turbulent passage through a static mixer. The resulting lipid vesicles, prior to an extrusion step, are in about 10% or more by weight ethanol, and have average diameter of from about 80 nm to about 200 nm.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an apparatus in accordance with the invention;

Fig. 2 shows an apparatus in accordance with the invention;

Fig. 3 shows a static mixer which may be used in the apparatus of Fig. 1 or Fig. 2;

Fig. 4 shows a custom designed continuous flow extruder;

Fig. 5 sets out a method of using liposomes prepared according to the invention.

Fig. 6 shows the influence of citrate buffer stream turbulence on vesicle size during continuous flow hydration;

Fig. 7 shows the influence of temperature on vesicle size during continuous flow hydration; and

Fig. 8 shows the influence of lipid concentration on spontaneous vesicle formation during continuous flow hydration.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

While the terms used in the application are intended to be interpreted with the ordinary meaning as understood by persons skilled in the art, some terms are expressly defined to avoid any ambiguity. Thus, as used in the specification and claims of this application the term:

charged lipid refers to a lipid species having either a cationic charge or negative charge or which is a zwitterion which is not net neutrally charged, and generally requires reference to the pH of the solution in which the lipid is found.

fully encapsulated refers to lipid particles in which the therapeutic agent is contained in the lumen of a lipid vesicle such as a liposome, or embedded within a bilayer of a lipid particle such that no part of the therapeutic agent is directly accessible to the external medium surrounding the lipid particle. Lipid particles in which the therapeutic agent is fully encapsulated are distinct from particles in which a therapeutic agent is complexed (for example by ionic interaction) with the exterior of the particle, or from particles in which the therapeutic agent is partially embedded in the lipid and partially exposed to the exterior medium. The degree of encapsulation can be determined using

methods which degrade available therapeutic agent. In the case of a polynucleotide, these methods include S1 Nuclease Digestion, Serum Nuclease, and Micrococcal Nuclease analysis. Alternatively, an OliGreen™ assay can be employed. In a quantitative sense, a "fully encapsulated" therapeutic agent is one where less than 10% of the therapeutic agent, and preferably less than 5% of the therapeutic agent in a lipid particle is degraded under conditions where greater than 90% of therapeutic agent is degraded in the free form. It should further be noted that additional therapeutic agent(s) may be associated with the lipid particle by complexation or another manner which is not fully encapsulated with out departing from the present invention.

hydration refers to a common process by which lipid particles, including liposomes, are formed. In this process, the amount of water in the solvent surrounding the lipids is increased from a concentration of around 5% or less (at which concentration the lipid molecules are generally individually solvated) to a concentration of 40-60 % or greater (at which lipids spontaneously form into membranes, micelles or particles).

lipid refers to a group of organic compounds that are esters of fatty acids and are characterized by being insoluble in water but soluble in many organic solvents. They are usually divided in at least three classes: (1) "simple lipids" which include fats and oils as well as waxes; (2) "compound lipids" which include phospholipids and glycolipids; and (3) "derived lipids" such as steroids. A wide variety of lipids may be used with the invention, some of which are described below.

Liposomes refers to vesicles having a self-closed structure of generally spherical or oval shape formed from one or more lipid layers and having an interior lumen containing a part of the solvent. Liposomes may be unilamellar, oligolamellar or multilamellar structures, unless specified.

The invention disclosed herein relates to novel methods and apparatuses for making lipid vesicles which are particularly applicable to the large-scale manufacture. The methods and apparatus employ a high flow rate static (motionless) mixer wherein hydration of lipid in an organic solvent takes place in a controlled fashion under highly turbulent conditions. Sizes of resulting particles can be selected by carefully selecting process parameters such as lipid and other solute concentrations, turbulence, temperature, volumetric ratios of mixing streams, etc. Since sizes are determined during the hydration

step, no post-hydration extrusion step is required to obtain desired size ranges of lipid vesicles.

This method has several important characteristics which make it of substantial utility to the art. First, it is a large-scale method which can be used to make substantial quantities (e.g. >1000 litres) of liposomes in a single run and in conjunction with diafiltration can be used in a continuous flow process. Second, the size of resulting liposomes can be selected in advance so that extrusion processing of the liposomes to reach a therapeutically desirable size is not necessary. Third, problems associated with previous hydration technologies, such as foaming and fluctuating concentrations of materials can be avoided.

In accordance with the invention, lipid vesicles are made by preparing a solution of lipid in an organic solvent, preferably ethanol, comprising from about 1 to about 100 mg/ml lipid. A second solution of hydration buffer, such as a standard pharmaceutical buffer or a standard liposome buffer (i.e. 300 mM citrate (pH 4.2)) is also prepared. By means of a carefully selected injection port (having a narrowly defined diameter of about 2 mm or less, preferably 0.25 to 1.0 mm), lipid in solvent (the "side stream") is injected into the buffer stream (the "main stream") at ratios and flow rates as detailed below. The two streams are mixed by a controlled highly turbulent passage through a static mixer, consisting of a length of pipe with sufficient mixing elements or other features which exploit fluid dynamics to achieve mixing. The result is liposomes having an average diameter of from about 80 nm to about 200 nm, in about 10% or more of the original organic (lipid) solvent.

Figs. 1 and 2 show two alternative embodiments of apparatus which can be used for preparation of empty lipid vesicles in accordance with invention using a

continuous flow hydration (mixing) procedure with a static mixer (or motionless mixer). These embodiments share certain common features, most importantly a first feeder tank for lipid in solvent, a second feeder tank for aqueous buffer, a combining chamber for receiving and combining outflow of the first feeder tank with outflow of the second feeder tank, a static mixer means for turbulent mixing of the product of the combining chamber; and a holding tank for collecting the product of the hydration process.

A suitable static mixer is the Statiflo Motionless Mixer (Statiflo Inc. Toronto). The static mixer design is set out in Fig. 3. Side stream **1** is injected into a receiving reservoir **2** through an injection port **3** of 2mm diameter or less. Port diameters of 1mm or 0.25 are preferred, and smaller port sizes are useable. The receiving reservoir is additionally disposed to receive main stream **4** and to direct the combining streams into the mixing domain **5.**

The static mixer employs the principles of radial momentum transfer, flow division and shear plane reversal. These transport phenomena combine to eliminate concentration, velocity and thermal gradients. By using an elliptical shape of mixing elements, smooth transitions are possible and no energy is wasted in back mixing. Thus, static mixer will completely blend and disperse two fluids in short lengths of piping. The mixing elements used are made in two patterns: a left-handed inclined ellipse (LH) provides clockwise rotational flow and the right-handed inclined ellipse (RH) provides counterclockwise rotational flow. The elements are connected at 90° angels to each other and the two element patterns are alternated in the following series: RH, LH, RH, LH, etc. In some designs, mixing elements create regions of relatively greater turbulence alternating with relaxation zones.

Because a static mixer operates in a pipeline, fluids proceed axially through the line in a flow regime defined by the degree of turbulence characterized by the dimensionless Reynolds number N_{re}. $N_{re} < 500$ is laminar flow; $500 < N_{re} < 2000$ is transitional; $N_{re} > 2000$ is fully turbulent. N_{re} dictates the flow regime and therefore determines how many mixing elements are necessary for a particular application. Approximately 4 – 6 mixing elements are suitable for satisfactory mixing in the examples hereto, when using lipid and therapeutic agents in the concentrations, volumes, mixing ratios, port sizes and flow rates employed.

Fully turbulent systems may also be prepared without the use of mixing

elements. Under suitable injection conditions and flow rates, fluid dynamics within a simple pipe, of sufficient length, width and construction can generate turbulence with N_{re} > 2000.

The main benefit of a static mixer is that when ethanolic lipid and buffer solution concentrations are constant, and flow rates are precisely metered, resulting particle sizes and characteristics can be precisely defined. Other benefits of static mixer in the invention include the following: Mixing conditions are constant throughout the procedure and concentration of the ethanol in the reaction cell is fixed over time; Batch size is unlimited since receiving tanks can be systematically filled; easy installation, operation and cleaning; absence of moving parts; cost effectiveness; limited energy loss, back mixing and redundant mixing; Adjustability of N_{re} and shear stress; long service life; reduced manpower requirements; and no external power source required.

The static mixer described above and depicted in Fig. 3 may be put into practice using the apparatus of Fig. 1 as set forth below.

Solutions to be mixed are prepared and stored in feeder tank **1** and feeder tank **2**. Generally these solutions are a lipid in organic solvent, such as ethanol, and an aqueous buffer. When mixing is desired, solutions are pumped via pump **3** into a static mixer **5**, described above. Flow rates, temperature, shear rate and many other parameters as set out in the examples below are carefully controlled. Liposomes form in the solution stream and are deposited into stainless-steel holding vessel **20** (volume 20 – 200 litres), maintained at 28 °C by thermal jacket **21**.

Though not required by this invention, if a post-loading extrusion step is desired, the prepared empty liposome solution in holding vessel **20** may be cycled through the optional continuous flow extrusion circuit **24**. Circuit **24** includes diaphragm-metering pump (Bran & Luebbe, Model: N-D31) **14**, a heat exchange system **10**, which raises the solution temperature to a temperature suitable for extrusion, generally 65°C, and extruder **9**. Extruder **9** is a custom designed continuous flow extruder set out in Fig. 4. This extruder includes two plates of 25 mm thickness, 316 SS and a secure closing system which allows use of pressures up to 1000 psi. The plates form an internal volume to 125 ml and surface area sufficient for a 142 mm membrane. Membranes are polycarbonate membranes having 142 mm diameter and preferably either 50nm, 80 nm or 100 nm pore size (Poretics, Inc. or Nucleopore, Inc.) though other sizes may be used. Two stacked

membranes are used for each pass. It is convenient to use two holding vessels, **11** and **20** to collect extruded material during these extrusion steps. The extruded material is transferred to lower reservoir **11** prior to each cycle to ensure the exact amount of passes for each extrusion circuit. Flow rate through the extrusion circuit is from 50 to 2000 ml/min.

The static mixer of Fig. 3 may also be employed in the apparatus of Fig. 2. The apparatus of Fig. 3 provides ethanolic lipid feeder tank **31** and buffer feeder tank **32.** A standard rotary pump **33** or a nitrogen pressure driven pump **34** can be used to sensitively adjust solution flow rates. Static mixer **35** receives both solutions, mixes them and directs the product to a large hydration tank (20 - 1000 litres) **36**. A head and tail collector tank **37** is useful to recover excess solution.

Liposomes prepared with the static mixer apparatus of either Fig 1 or Fig 2 require final processing before being used in therapeutic applications. For final processing, the suspension of liposomes is dialyzed, such as by tangential flow dialysis or diafiltration, to replace buffer and remove unwanted components, and liposomes are concentrated. The replacement buffer is a traditional pharmaceutically acceptable buffer such as Phosphate Buffer Solution (PBS) (pH 7.4). Unwanted components include ethanol, unencapsulated lipids or initial buffer. Fig. 1 also describes a diafiltration and concentration system. Typically, diafiltration systems include hollow fiber cartridge(s) **21** (UFP-100-C-55 (100,000 mw cut-off; 3.2m^2), A/G Technology Corp.), which may be used in parallel to increase hollow fiber surface area and consequently permeate flow rate. The circuit also includes sanitary rotary lobe pump **20** (Lobtop 350, Teknoflow, Inc.), process vessel **18** (Polysulfone, vol. 5L, A/G Technology Corp), buffer tank **13** (vol. 50 L, polypropylene, Nalgene) and 316 stainless steel flexible tubing (Inland Machinery). The suspension is circulated through the ultrafiltration column under low pressure (10 psi), and permeate is driven out. Ethanol and unwanted lipids or buffer below approx 8,000 MW should be fully removed in permeate. Replacement buffer for diafiltration, such as PBS, flows from tank **13** according to the vacuum created by the diafiltration process. Permeate may optionally be collected for recovery of components of the system. Typical permeate flow rate during diafiltration , for one cartridge (3.2m^2) is 1.0-1.4 L/min. Temperature during diafiltration is 26-28 °C. A de-foaming circuit, including vessel **17** may also be employed in the diafiltration circuit.

Once the buffer is replaced and unwanted components are removed, the suspension is ready for final concentration. This is achieved by continuing the circuit without the addition of replacement buffer. This system routinely concentrates a 5 mg/ml solution to 15 mg/ml for packaging and distribution. Final concentrated product is collected and stored in pharmaceutically acceptable plastic, glass or stainless steel to await sterile filtration and packaging according to methods known in the art.

Fig. 2 also depict the diafiltration and concentration apparatus as follows: process tank **37**, diafiltration vessels **38**, sanitary rotary lobe pump **40** (Lobtop 350, Teknoflow, Inc.), process and concentration vessel **39** (Polysulfone, vol. 5L, A/G Technology Corp), buffer tank **32** (vol. 50 L, polypropylene, Nalgene) and 316 stainless steel flexible tubing (Inland Machinery).

Having set out a general description of the methods and apparatus of the invention, further particulars are now described.

Preparation and Selection of Lipids

The liposomes of the present invention generally consist of a combination of several types of lipids Specific lipid components may be selected from among the following non-limiting examples.

Charged Lipids

A wide variety of charged lipids may be used with the invention.

Cationic charged lipids at physiological pH include, but are not limited to, N,N-dioleyl-N,N-dimethylammonium chloride ("DODAC"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTMA"); N,N-distearyl-N,N-dimethylammonium bromide ("DDAB"); N-(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride ("DOTAP"); 3β-(N-(N',N'-dimethylaminoethane)-carbamoyl)cholesterol ("DC-Chol") and N-(1,2-dimyristyloxyprop-3-yl)-N,N-dimethyl-N-hydroxyethyl ammonium bromide ("DMRIE"). Additionally, a number of commercial preparations of cationic lipids are available which can be used in the present invention. These include, for example, Lipofectin™ (commercially available cationic liposomes comprising DOTMA and 1,2-dioleoyl-sn-3-phosphoethanolamine ("DOPE"), from GIBCO/BRL, Grand Island, New York, USA); Lipofectamine™ (commercially available

cationic liposomes comprising N-(1-(2,3-dioleyloxy)propyl)-N-(2-(sperminecarboxamido)ethyl)-N,N-dimethylammonium trifluoroacetate ("DOSPA") and DOPE from GIBCO/BRL); and Transfectam™ (commercially available cationic lipids comprising dioctadecylamidoglycyl carboxyspermine ("DOGS") in ethanol from Promega Corp., Madison, Wisconsin, USA).

Some cationic charged lipids are titrateable, that is to say they have a pKa at or near physiological pH, with the significant consequence for this invention that they are strongly cationic in mild acid conditions and weakly (or not) cationic at physiological pH. Such cationic charged lipids include, but are not limited to, N-(2,3-dioleyloxy)propyl)-N,N-dimethylammonium chloride ("DODMA") and 1,2-Dioleoyl-3-dimethylammonium-propane ("DODAP").

Anionic charged lipids at physiological pH include, but are not limited to, phosphatidyl inositol, phosphatidyl serine, phosphatidyl glycerol, phosphatidic acid, diphosphatidyl glycerol, poly(ethylene glycol)-phosphatidyl ethanolamine, dimyristoylphosphatidyl glycerol, dioleoylphosphatidylethanolamine (DOPE), dioleoylphosphatidyl glycerol, dilauryloylphosphatidyl glycerol, dipalmitoylphosphatidyl glycerol, distearyloylphosphatidyl glycerol, dimyristoyl phosphatic acid, dipalmitoyl phosphatic acid, dimyristoyl phosphatidyl serine, dipalmitoyl phosphatidyl serine, brain phosphatidyl serine, and the like.

Some anionic charged lipids may be titrateable, that is to say they would have a pKa at or near physiological pH, with the significant consequence for this invention that they are strongly anionic in mild base conditions and weakly (or not) anionic at physiological pH. Such anionic charged lipids can be identified by one skilled in the art based on the principles disclosed herein.

Neutral Lipids and sterols

The term "neutral lipid" refers to any of a number of lipid species which exist either in an uncharged or neutral zwitterionic form a physiological pH. Such lipids include, for example, diacylphosphatidylcholine, diacylphosphatidylethanolamine, ceramide, sphingomyelin, cephalin, cholesterol, cerebrosides and diacylglycerols.

Modified Lipids

Certain preferred formulations used in the invention include aggregation preventing lipids such as PEG-lipids or polyamide oligomer-lipids (such as an ATTA-lipid), and other steric-barrier or "stealth"-lipids. Such lipids are described in US Patent Nos. 4320121 to Sears, 5,820,873 to Choi et al., 5,885,613 to Holland et al., WO 98/51278 (inventors Semple et al.), and US Patent Application Serial No. 09/218988 relating to polyamide oligomers, all incorporated herein by reference. These lipids prevent precipitation and aggregation of formulations containing oppositely charged lipids and therapeutic agents. These lipids may also be employed to improve circulation lifetime *in vivo* (see Klibanov et al. (1990) FEBS Letters, 268 (1): 235-237), or they may be selected to rapidly exchange out of the formulation *in vivo* (see US Pat. No. 5885613). Particularly useful exchangeable lipids are PEG-ceramides having shorter acyl chains (i.e, C_{14} or C_{18}, referred to herein as PEG-CerC_{14} and PEG-CerC_{18}) or PEG-PE having a C_{14} acyl chain.

Some lipid formulations may employ targeting moieties designed to encourage localization of liposomes at certain target cells or target tissues. Targeting moieties may be linked to the outer bilayer of the lipid particle during formulation or post-formulation. These methods are well known in the art. In addition, some lipid formulations may employ fusogenic polymers such as PEAA, hemagluttinin, other lipo-peptides (see US Patent applications SN 08/835,281, and 60/083,294, all incorporated herein by reference) and other features useful for *in vivo* and/or intracellular delivery.

Solvents and Lipid Combinations

Many combinations of lipids may be employed to make liposomes of the invention. Generally, these combinations are formulated in the lipid feed stock solution and mixed to provide a highly homogeneous solution. Care should be taken to employ solvents or solubilizing agents (such as detergents) in which selected lipid combinations will dissolve, together, at desired concentrations. A wide range of solvents and solubilizing agents may be employed. Preferred organic solvents are set out below. An alternative method employs detergents, and the like, which can solubilize lipids in aqueous solvent. Detergents may be preferred in certain circumstances, for instance, if lipids are not sufficiently soluble in the desired organic solvent.

For purpose of this specification, "organic solvent" means either a completely organic solvent (i.e. 100% ethanol) or a partially organic solvent (such as ethanol in water, i.e. 20% ethanol, 40% ethanol, etc.). A wide variety of water miscible organic solvents may be used including ethanol or other alcohols, acetonitrile, dimethylformamide, DMSO, methylene chloride, chlorofluorocarbons, acetone, other ketones, and the like. Solvents with greater or lesser polarity may be useful in some cases.

Detergent solutions include β-D-glucopyranoside, Tween 20 and those set out in WO 96/40964 and US Patent application SN 09/169573, both incorporated herein by reference, and any other detergent or steric barrier compound that can provide the same solubility features, and/or can prevent particle aggregation during formulation.

Preferably all organic solvents or detergent solutions are pharmaceutically acceptable in trace amounts in order that residuals remaining from the formulation process do not preclude patient administration.

Many types of lipid combinations may be formed into liposomes using the methods and apparatuses of the invention. Those skilled in the art will recognize both prior art combinations and novel combinations of lipids can be formulated. Typical prior art formulations are standard EggPC/Chol, DSPC/Chol or PEG-PE/DSPC/Chol and the like. Particularly preferred are sphingosomes comprised of sphingomyelin and cholesterol, the subject of US Pat. No. 5,543,152 incorporated herein by reference. Widely varying molar ratios of lipids may be employed.

Novel lipid vesicles, particularly lipid vesicles comprising cationic or anionic charged lipids may be prepared. A useful cationic lipid vesicle for use with nucleic acid therapeutics, which is the subject of PCT Patent Publication WO 98/51278 of Semple et al. comprises the following amounts of the following lipid components: 10 to 40 mol % charged lipid; 25 to 45 mol% neutral lipid, 35-55 mol% sterol; and 0.5 to 15 mol % modified lipid (such as a PEG-lipid).

Continuous Flow Hydration is found to be very sensitive to lipid concentration of the ethanolic lipid "side stream". Suitable ranges of lipid feed stock concentrations range from 1 mg/ml to 100 mg/ml. Preferred lipid feed stocks are 5-25 mg/ml. Most preferred for sphingomyelin/cholesterol formulations are lipid feed stocks in the range of 10 - 20 mg/ml.

The potential of using low lipid feeder stock concentrations of 1-25 mg/ml provides a major advantage of the present invention over prior art methods because it expands the types of lipids and types of solvents which may be employed. Previously, combinations of lipids were limited by the requirement that all must be soluble to the desired (and high) degree in the same solvent. The present invention now permits use of lipids which are only slightly soluble in solvents preferred by the user, because only a low total concentration needs to be achieved.

Hydration Buffer

Hydration buffer is supplied in the main stream of the apparatus. A wide variety of hydration buffers are suitable for mixing with the lipid feeder stock. A preferred hydration buffer is 300 mM citrate buffer (pH 4.2) because this may later be employed for loading liposomes with therapeutic agent (see infra). Alternative buffers such as phosphate buffered saline, normal saline, and the like, may also be used. Again, buffer must be pharmaceutically acceptable, as traces may remain in the final formulation.

Injection, Flow Rates and Turbulence

Examples below set out the effects of varying the key mixing parameters of the invention: the injection process, the flow rates and volumetric ratios of the side stream and main stream and the turbulence generated in the static mixer. A general theory of this invention, which explains why these parameters influence the size distribution of resulting liposomes, may be that amounts of lipid sufficient to assemble into a bilayer phospholipid fragment (BPF, *see* Lasic, D. 1988) of desired size must be injected in a quantum unit into hydration buffer. BPFs must be forced to self-assemble into liposomes of the desired size range. Final size is determined by the chance interactions of BPFs, which according to this invention, can be driven by turbulence and availability of BPFs (i.e. the concentration of BPFs in the mixing streams).

These results can also be explained by a spontaneous liposome formation model based on bilayered phospholipid fragments (BPF) as highly unstable transition structures formed during the initial stages of lipid hydration. When phospholipid/ethanol solution is injected into an aqueous phase, conditions are created where bilayered

phospholipid fragments (BPF) are formed. Their size is controlled by the injected lipid concentration and mixing efficiency. The free-floating BPF are thermodynamically unstable because the non-polar hydrocarbon chains at their edges are exposed to water. The BPF reduce and finally eliminate this unfavorable interaction by bending and closing upon themselves. In this case, the size and structure of the vesicles depends on the lamellarity and size of BPF and the SUVs formed by fusion of the fragments.

Continuous flow hydration technology, sometimes called "precision-metered hydration", allows lipid hydration to be performed under constant conditions of lipid concentration, ethanol concentration, temperature and mixing. For this process, any flow chamber that allows simultaneous mixing of two or more different fluid flows can be used. The simplest system uses a static mixer. Static (motionless) mixers exhibit efficient agitation and low or moderate shear rate. The turbulence of the buffer stream and mixing elements result in effective mixing. Smaller and more uniform vesicles can be obtained compared to conventional injection techniques. Under the flow and turbulence conditions employed in the invention, the major fraction of the liposomes formed can be expected to be LUV (60-130nm). Using this model, we can also explain the influence of lipid concentration on size and lamellar structure of vesicles during continuous flow hydration.

Techniques found to be useful for making the preformed lipid vesicles include the use of a static mixer. Any method may be employed, but the method will effect the size of the empty liposome, shown in one experiment, approximately as follows:

Method	Median Empty Liposome Size
Static Mixer (Low Turbulence)	150-170 nm
Static Mixer (High Turbulence)	100-120 nm

Other examples are set out in the Examples, below.

Preferred sizes for liposomes made by the various liposome sizing methods will depend to some extent on the application for which the liposome is being made, but will in general fall within the range of 25 to 250 nm. Specific examples of suitable sizes are set out in the Examples below.

It has been observed that there are at least 5 major factors which define vesicle size, structure of liposomes (MLV or ULV) and entrapped volume of liposomes resulting from the methods and apparatuses of the invention:

1. Lipid composition (including Phospholipid/Cholesterol ratio)
2. Lipid concentration
3. Organic solvent (ethanol)
4. Temperature during hydration
5. Mixing turbulence

General descriptions of these parameters are set out below, and specific example illustrated in the examples section.

Optional Sizing of Lipid Particles

In general, a sizing step of the type known in the art is not necessary. Should sizing of the liposomes be desired, however, an optional step for sizing of liposomes may be employed. There are several methods for the sizing of lipid particles, and any of these methods may generally be employed.

The extrusion method is a preferred method of liposome sizing. *see* Hope, MJ et al. Reduction of Liposome Size and Preparation of Unilamellar Vesicles by Extrusion Techniques. In: Liposome Technology (G. Gregoriadis, Ed.) Vol. 1. p 123 (1993). The method consists of extruding liposomes through a small-pore polycarbonate membrane or an asymmetric ceramic membrane to reduce liposome sizes to a relatively well-defined size distribution. Typically, the suspension is cycled through the membrane one or more times until the desired liposome size distribution is achieved. The liposomes may be extruded through successively smaller pore membranes to achieve gradual reduction in liposome size.

A variety of alternative methods known in the art are available for reducing the size of a population of liposomes ("sizing liposomes"). One sizing method is described in U.S. Patent No. 4,737,323, incorporated herein by reference. Sonicating a liposome suspension either by bath or probe sonication produces a progressive size reduction down to small unilamellar vesicles less than about 0.05 microns in diameter. Homogenization is another method; it relies on shearing energy to fragment large liposomes into smaller ones. In a typical homogenization procedure, multilamellar vesicles are recirculated through a standard emulsion homogenizer until selected liposome sizes, typically between about 0.1 and 0.5 microns, are observed. The size of the liposomal vesicles may be determined by quasi-electric light scattering (QELS) as

described in Bloomfield, Ann. Rev. Biophys. Bioeng., 10:421-450 (1981), incorporated herein by reference. Average liposome diameter may be reduced by sonication of formed liposomes. Intermittent sonication cycles may be alternated with QELS assessment to guide efficient liposome synthesis.

Uses of Empty Liposomes

There are many ways in which the empty liposomes of the invention may be employed. There are certain conventional techniques, and certain novel techniques currently being developed.

In a conventional technique, the empty liposomes may be loaded with therapeutic agent by an ionic or pH gradient as described in U.S. Pats. Nos. 5,785,987, 5,380,531 , 5,316,771 and 5,192,549. Alternatively, the empty liposomes may be used alone, for therapeutic applications such as in the invention of Williams US Pat. No. 5,858,400. There are many ways known in the art to employ liposomes of the size and quality produced by the methods and apparatuses herein.

Fig. 5 illustrates one embodiment by which liposomes of this invention may be used. Empty liposomes according to the invention are prepared in 40% ethanol and are placed in a reservoir to which a therapeutic agent, such as a therapeutic oligonucleotide is added. Quite surprisingly, and by a mechanism of membrane interaction yet to be fully understood, a very high level of oligonucleotide is found to be encapsulated within the lipid particle after mixing (drug:lipid ratio = 0.1 to 0.2). Efficiency of encapsulation is also very high, with 60-90% of the starting oligonucleotide being encapsulated in final, patient administration-ready particles. While not intending to be bound by any particular mechanistic theory, it is noted that existing models emphasize the effects of membrane dynamics where oppositely charged particles cause a shift in membrane stresses which cause interaction of the empty preformed vesicles, thus encapsulating oligonucleotides that would otherwise be on the outside of the vesicle. Oligonucleotides are not thought to be able to permeate a lipid membrane. This technique is the subject of concurrently filed PCT Application entitled "Methods for Preparation of Lipid-encapsulated Therapeutic Agents, filed 14 July 2000, Serial number not yet

assigned, attorney docket no. 80472-7, based on the same priority application as the instant patent application, which is incorporated herein by reference.

The following examples set out specific, non-limiting embodiments of the invention claimed below.

EXAMPLES

Example 1

This example illustrates a standard trial run of continuous flow hydration with static mixer (non-extrusion technology), using the apparatus of this invention, for production of Sphingomyelin/Cholesterol liposomes of 80-130 nm average diameter.

Materials: (Grade/Type/IDC Lot No) (Source): Sphingomyelin (100% purity (MS0043)) (Lipoid); Cholesterol (>95% purity (MS008-0001)) (Solvay); Ethanol (100%) (Commercial Alcohol Inc, Toronto); Citric Acid Monohydrate (USP)(J.T. Baker); Sodium citrate Dihydrate (USP) (J.T. Baker); Milli Q water (Millipore)

Equipment list: Static mixer Statiflo100 (6 mixing elements, 1or 2 (1mm) injection ports) (Watt-Pearson Ltd.); Submicron Particle Sizer Model 370 (NICOMP Particle Sizing system, Santa Barbara); MasterFlex Peristaltic pump Model 7523-20 (#D98003335); Pump head Model 7518-12; Rotary-Lobe Pump Labtop 350 rotary lobe pump (A/G Technology Corp., Needham, MA); Membrane cartridge M15S-260-01N (Spectrum Microgon, laguna Hills, CA)

The hydration procedure employed the following parameters:

Batch size (after hydration) = 2000 ml
Ethanol concentration after hydration = 15 %
Sphingomyelin/Cholesterol formulation (Approximately 55:45 %)
Total lipid concentration after hydration = 2.0 mg/ml
Concentration of the lipid stock solution = 13 mg/ml

The main stream flow applied = 4400 ml/min
One 1mm injector
The side stream injection flow = 775 ml/min
Hydration temperature = RT

Ultrafiltration was performed as follows: QuixStand system and membrane cartridge M15S-260-01N or UFP-100-C-4A (A/G Technnology Corp) was used for first concentration step. MidGee system membrane cartridge M15S-260-01N or UFP-100-C-4A (A/G Technnology Corp) was used to perform diafiltration with 300mM CBS (pH=3.95) and for final concentration. Diafiltration employed 10-15 wash volumes of CBS.

Results are set out in **Table 1:** Standard Sizing Run

Note: The apparent change in liposome size before and after diafiltration and concentration is artifactual. Post-hydration NICOMP measurements are likely identifying non-specific and temporary associations between liposomes which have been stored at room temperature in ethanolic buffer having a low dialectric constant for several hours before reading. Typically, in continuous flow hydration, hydration is followed immediately by diafiltration and concentration. Diafiltration and concentration are not extrusion techniques and are not expected to fundamentally alter particle size.

Example 2

The reproducibility of the continuous flow hydration process using the methods and apparatus of the invention is demonstrated in the following experiments.

Lipid/Ethanol solution containing 10-25mg/ml sphingomyelin/cholesterol in molar proportions of approximately 55:45% was injected into an excess of 300mM Citrate buffer solution pH 4.0 using continuous flow hydration and motionless mixer with six helical elements (length - 17.7cm, ID - 1.6cm). During hydration, the lipid/ethanol solution was injected into the receiving reservoir through one or two injectors with aperture 0.5-1mm.

The main stream flow (300mM Citrate buffer solution pH 4.0) was 3200-5800ml/min; the side stream flow (Lipid/Ethanol solution) was 320-990ml/min. All experiments were performed at room temperature.

Table 2 demonstrates the reproducibility of continuous flow hydration for spontaneous vesicles formation {Experiments # 80, 81, 83 and 84 (Ethanol concentration after hydration 15%), Experiment # 82 (Ethanol concentration after hydration 20%)}.

Results are set out in **Table 2**: Reproducibility of the continuous flow hydration for vesicle formation

Example 3

This example demonstrates the reproducibility of the invention using two different alternative lipid formulation 1) Egg Phosphatidylcholine / Cholesterol and 2) Soya Phosphatidylcholine/Cholesterol

Lipid/Ethanol solution containing 10-25mg/ml Egg PC (Solvay)/Cholesterol or Soya Phosphatidylcholine (Lipoid)/Cholesterol, as indicated, in molar proportions of approximately 55:45% was injected into an excess of 300mM Citrate buffer solution pH 4.0 using continuous flow hydration and motionless mixer with six helical elements (length - 17.7cm, ID - 1.6cm). The main stream flow (300mM Citrate buffer solution pH 4.0) was 3200-8000ml/min, the side stream flow (Lipid/Ethanol solution) was 320-640ml/min. All experiments were performed at room temperature.

Table 3 demonstrates the reproducibility of continuous flow hydration for spontaneous unilamellar vesicle formation for a EggPC/Cholesterol formulations {Experiments # 11 (Ethanol concentration after hydration 10%), # 72 (#1-ethanol concentration after hydration 10%, #2- ethanol concentration after hydration 20%),and for Soya PC/Cholesterol formulations experiments # 85 and # 86 (Ethanol concentration after hydration 15%).

Example 4

This experiment demonstrates the effect of changes to the basic technological parameters of continuous flow hydration on vesicle size and other matters. Experiments were conducted as in the previous examples, using the same apparatus; but varying the technological parameters as indicated in each experiment.

Table 4: Influence of Sphingomyelin/Cholesterol ratio on vesicle size at different ethanol concentrations

Fig. 6: Influence of Citrate buffer stream turbulence on vesicle size during continuous flow hydration

Note: Laminar mainstream flow was 250 ml/min (Nre = 312<500)
Transitional mainstream flow was 1700 ml/min (Nre = 500<1935<2000)
Turbulent mainstream flow rate was 3200 ml/min (Nre = 3200>2000)
Lipid concentration after hydration 10mg/ml, ethanol concentration 10%

Fig. 7: Influence of temperature on vesicle size during continuous flow hydration

Note: Lipid concentration after hydration 10mg/ml and ethanol concentration 10%

Fig. 8: Influence of Citrate buffer stream turbulence on vesicle size during continuous flow hydration

Fig. 9: Influence of lipid concentration on spontaneous vesicle formation during continuous flow hydration (Nre - 3181-3636, injector ID -1mm)

Example 5

This example confirms that liposomes formed by the methods and apparatus of the invention are suitable for use as therapeutic agents, such as the liposomal vincristine.

Results are set out in **Table 5**:Loading efficiency for liposomes produced by continuous flow hydration

Example 6

The formulation and use of cationic liposomes according to the invention is now described.

Materials: Distearoylphosphatidylcholine (DSPC), was purchased from Northern Lipids (Vancouver, Canada). 1,2-dioleoyloxy-3-dimethylammoniumpropane (DODAP or AL-1) was synthesized by Dr. Steven Ansell (Inex Pharmaceuticals) or, alternatively, was purchased from Avanti Polar Lipids. Cholesterol (CHOL) was purchased from Sigma Chemical Company (St. Louis, Missouri, USA). PEG-ceramides were synthesized by Dr. Zhao Wang at Inex Pharmaceuticals Corp. using procedures described in PCT WO 96/40964, incorporated herein by reference. [^{3}H] or [^{14}C]-CHE was purchased from NEN (Boston, Massachusetts, USA). All lipids were > 99% pure. Ethanol (95%), methanol, chloroform, citric acid, HEPES and NaCl were all purchased from commercial suppliers. Lipid stock solutions were prepared in 95% ethanol at 20 mg/mL (PEG-Ceramides were prepared at 50 mg/mL).

The four lipids were dissolved in a 100% ethanol to a total lipid concentration of 25 mg/ml (33 mM). The ethanolic lipid was then introduced through an injection port with an orifice diameter of 0.25 mm using the apparatus of Fig. 2 and combined with 300 mM citrate buffer, pH 4.0. The total volume of ethanolic lipid was 6 liters, and the flow rate for lipid introduction was 200-300 ml/min. The total volume of citrate buffer was 9 liters. The resulting 15 liter mixture had an ethanol concentration of 40% and 180 mM citrate . Vesicles of 90-120 nm median diameter were generated. The empty preformed vesicles were then pooled in reservoir of the apparatus of Fig. 2 and maintained at 40°C until addition of therapeutic agent solution.

Oligonucleotide particles were then made using empty preformed vesicles prepared using the static mixer process from a lipid mixture containing PEG-CerC14, DODAP, DPSC and CHOL in a molar ratio of 5:25:25:45. This procedure takes advantage of the remarkable finding that preformed empty liposome vesicles, will spontaneously encapsulate oligonucleotides when mixed with the buffered oligonucleotide solution (the subject of concurrently filed PCT Patent Application S.N._____ which relies on the same priority document as the instant invention.) Preformed vesicles were used to make fully lipid-encapsulated therapeutic agent particles using oligonucleotide INX-6295 (a c-myc antisense ODN with the sequence

5'-TAACGTTGAGGGGCAT-3' Seq. ID No. 1) as the therapeutic agent. Oligonucleotide INX-6295 in distilled water was diluted by the addition of 100 % ethanol to form a various solutions of 10, 20, 30 40 or 50 mg/ml oligonucleotide in 40% ethanol. The ethanolic oligonucleotide was added to the preformed vesicles in reservoir 20 at 40°C with gentle mixing. The amount and volume of ethanolic oligonucleotide was calculated to provide a final drug:lipid ratio of 0.1 to 0.25 by weight. The mixture was then incubated at 40°C with gentle and periodic mixing for 1 hour. After incubation, the solution was processed by diafiltration to strip free or excess associated oligonucleotide, remove ethanol and exchange the buffer system to phosphate buffered saline (PBS), pH 7.4. Concentration, sterile filtration and packaging complete the preparation of a commercial product.

Table 1

	Vesicle Size (nm)		Total Lipid				
Batch	Post Hydration	Post Diafiltration	Stock mg	Final Product Conc mg/ml	Final Product mg	Wash Solution mg	Yield %
VSLI-24	96	84	2941	103	2051	468	70
VLSI-88/1	115	118	6000	88	4050	500	76
VLSI-88/2	109	111	6000	104	4650	300	82

Table 2: Reproducibility of the continuous flow hydration for vesicle formation

Exp	run	Main stream Flow (ml/min)	Side stream flow (ml/min)	Flow ratio	Reynolds Number $N_{re\,(SM)}$	Linear velocity (m/s)	Shea rate (s^{-1})	Post hydration	
								Vesicle Size nm	Std. Deviation %/Chi Squared
80	3	3350	570	5.9	3815	0.27	135.0	122	32/0.23
	4	3350	570	5.9	3815	0.27	135.0	129	35/0.38
	5	3400	590	5.76	3872	0.28	141.0	129	34/0.35
81	6	5750	990	5.8	6553	0.48	240.0	115	29/0.29
	7	5750	990	5.8	6553	0.48	240.0	114	31/0.47
82	8	4000	990	4.0	4557	0.33	166.0	140	33/0.33
	9	4000	990	4.0	4557	0.33	166.0	145	33/0.35
83	10	5650	990	5.74	6424	0.47	235.0	124	36/0.23
	11	5650	990	5.74	6424	0.47	235.0	124	30/0.35
	12	5650	990	5.74	6424	0.47	235.0	136	35/0.20
	13	5650	990	5.74	6424	0.47	235.0	138	34/0.55
84*	14	5650	990	5.74	6424	0.47	235.0	136	32/017
	15	5650	990	5.74	6424	0.47	235.0	146	36/0.26
	16	5650	990	5.74	6424	0.47	235.0	144	37 /0.21

*) No mixing elements applied during hydration procedure

Table 3: Reproducibility of continuous flow hydration for vesicle formation

Exp		Main stream flow (ml/min)	Side stream flow (ml/min)	Flow ratio	Reynolds Number $N_{re\,(SM)}$	Shear Rate (s^{-1})	Post hydration	
							Vesicle Size nm	Std. Deviation %
11	2	2800	300	9	3181	116	149	49

	4	4800	300	16	5455	199	137	40
	8	8000	300	27	9091	332	135	39
7 2	1	3200	320	5	3636	133	167	45
	2	3200	640	10	3636	133	106	40
85 (*)	1	5600	970	5.7	6379	232.0	98	49
	2	5600	970	5.7	6379	232.0	98	52
8 6	1	5600	970	5.7	6379	232.0	105	53
	2	5600	970	5.7	6379	232.0	118	58
	3	5600	970	5.7	6379	232.0	103	47

Table 4: Influence of Sphingomyelin/Cholesterol ratio on vesicle size at different ethanol concentrations

	Sphingomyelin/Cholesterol ratio			
Parameters	59/41 75/29mg/ml	58/42 71/29mg/ml	57/43 69/29mg/ml	55/45 65/29mg/ml
Ethanol 10% Injector 1mm	174nm	169nm	161 nm	168nm
Ethanol 17% Injector 1mm	185nm	138 nm	124 nm	120nm
Ethanol 10% Injector 0.5mm	116nm	N/A	N/A	N/A

Table 5: Loading efficiency for liposomes produced by continuous flow hydration

Sample	Vesicle size Before loading nm	Vesicle size After loading nm	Total vincristine mg/ml	Free vincristine mg/ml	Encapsulated Vincristine %
VSLI 88#1	118	119	0.164	0.008	95
VSLI 88#2	118	119	0.166	0.008	95

CLAIMS

1. An apparatus for preparation of a lipid vesicles comprising:

(a) a first reservoir for receiving a buffer composition;

(b) a static mixer for agitating buffer composition in the first reservoir;

(c) a second reservoir for receiving a lipid solution;

(d) a dispensing head for introducing lipid solution from the second reservoir into the first reservoir, and

(e) a connector joining the second reservoir to the dispensing head for conducting lipid solution from the second reservoir to the dispensing head, wherein the dispensing head has formed therein one or more injection ports having a diameter of 2 mm or less.

2. The apparatus of claim 1, wherein the first reservoir contains a citrate buffer and the second reservoir contains an ethanolic lipid solution comprising from about 1 to about 100 mg/ml lipid in at least 90% by weight ethanol.

3. The apparatus of claim 1 or 2, wherein the dispensing head has a plurality of injection ports formed therein.

4. The apparatus of claim 3, wherein the dispensing head has twenty or more injection ports formed therein.

5. A method of making lipid vesicles comprising:

(a) preparing a solution of ethanolic lipid comprising from about 1 to about 100 mg/ml lipid in at least 90% by weight ethanol;

(b) injecting the ethanolic lipid directly into aqueous buffer through an injection port of diameter about 2 mm or less to make a lipid/buffer mixture; and

(c) mixing the lipid/buffer mixture by turbulent passage through a static mixer to produce lipid vesicles, wherein the resulting lipid vesicles, prior to any extrusion

step, are in about 10% or more by weight ethanol, and have average diameter of from about 80 nm to about 200 nm.

6. The method of claim 5, wherein the buffer has pH below 5.0

7. The method of claim 5 or 6, wherein the buffer has a concentration of divalent cation greater than about 100 mM.

8. The method of any of claims 5-7, wherein the ethanolic lipid comprises sphingomyelin

9. The method of any of claims 5-7, wherein the ethanolic lipid comprises cholesterol

10. The method of any of claims 5-8, wherein the ethanolic lipid comprises sphingomyelin and cholesterol in a ratio by weight of from about 1:4 to about 4:1.

11. The method of claim 5, wherein the ethanolic lipid comprises a PEG-lipid conjugate, a cationic lipid, and a neutral lipid.

12. The method of claim 11, wherein the lipid in the ethanolic lipid comprises a PEG-lipid conjugate, a cationic lipid, a neutral lipid, and cholesterol in a ratio by weight of about 5: 25:25:45.

13. The method of claim 5, wherein the concentration of lipid in the ethanolic lipid is less than about 50mM.

14. The method of claim 5, wherein the concentration of lipid in the ethanolic lipid is about 1 to 20 mg/ml.

15. The method of any of claims 5-14, wherein said turbulence, measured by Nre, is greater than about 2000.

16. The method of claim 15, wherein said turbulence, measured by Nre, is greater than 5000.

17. The method of claim 5, wherein said average diameter is from about 100 nm to about 130 nm.

18. An apparatus for making empty unilamellar liposomes comprising:

(a) a first feeder tank containing an ethanolic lipid;

(b) a second feeder tank containing an aqueous buffer;

(c) a combining chamber disposed to receive and combine outflow of the first feeder tank with outflow of the second feeder tank; and

(d) a static mixer means for mixing the product of the combining chamber to form unilamellar lipid vesicles in 5 - 50% ethanol, said vesicles, prior to any extrusion, having average diameter of from about 80 nm to about 200 nm.

19. The apparatus of claim 18, further comprising

e) a reservoir for receiving the product of the static mixer means; and

f) a continuous flow extrusion circuit operably connected to the reservoir.

20. The apparatus of claim 18, further comprising

e) a reservoir for receiving the product of the static mixer means; and

f) a dialysis or diafiltration system operably connected to the reservoir.

21. The apparatus of any of claims 18-21, wherein the Nre of the static mixer means is > 2000.

22. The apparatus of any of claims 18-21, with the proviso that no continuous flow solvent removal means is incorporated with elements c) through e).

23. The apparatus of any of claims 18-22, wherein the combining chamber comprises an injection port of diameter 2 mm or less.

24. The apparatus of any of claims 23-27, wherein the combining chamber combines the outflow of the first feeder tank with outflow of the second feeder tank in a volumetric ratio of from about 1:20 to about 2:1.

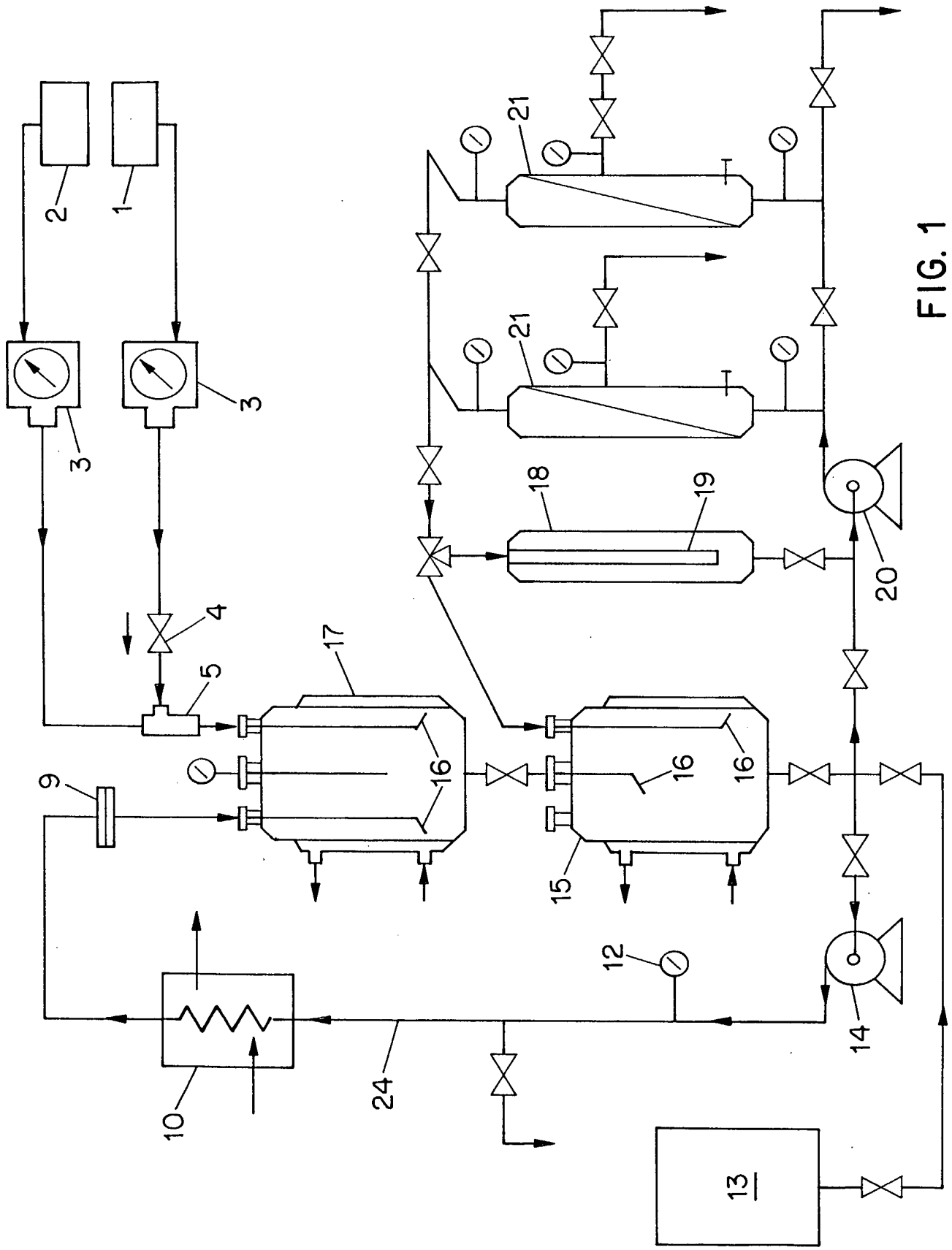


FIG. 1

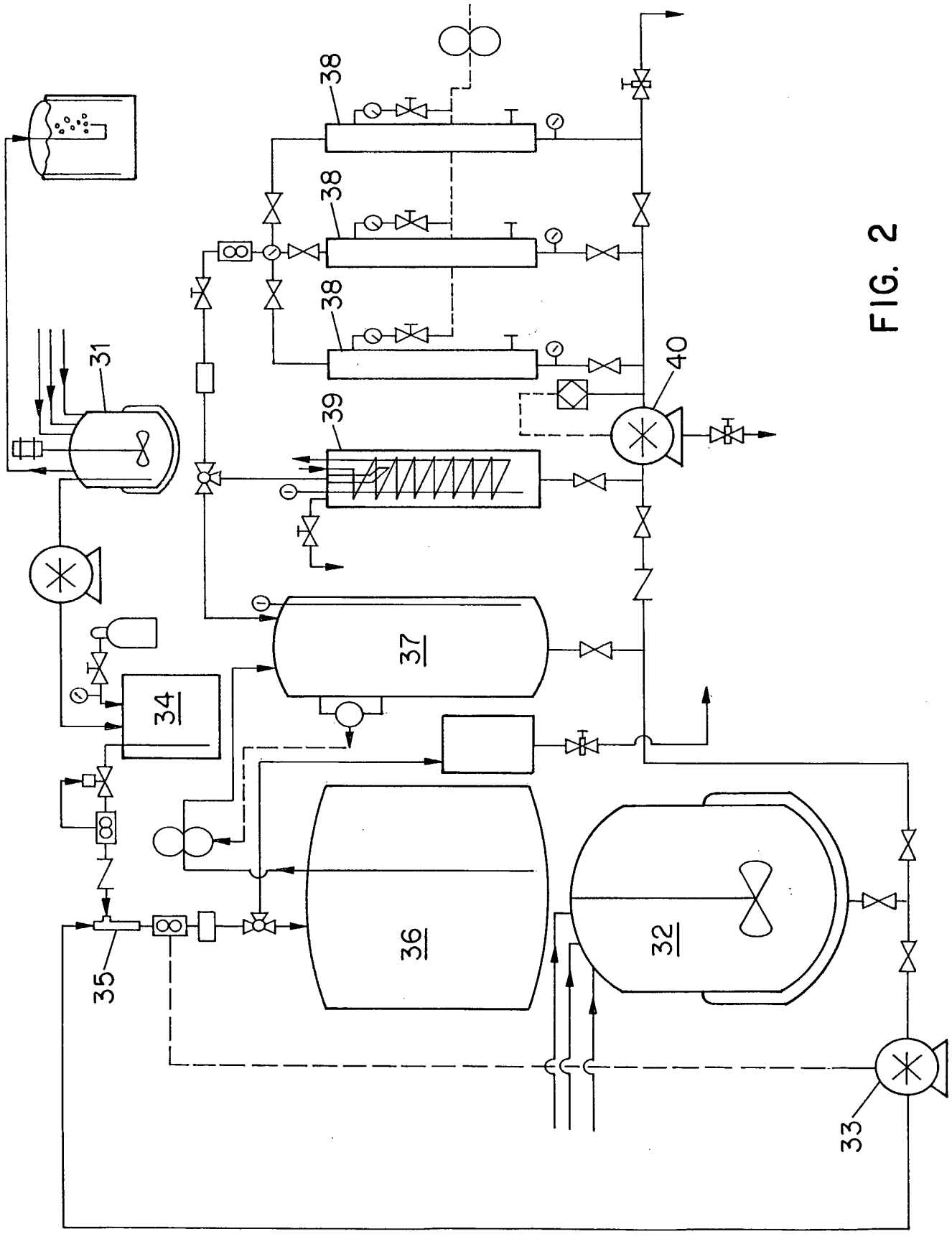
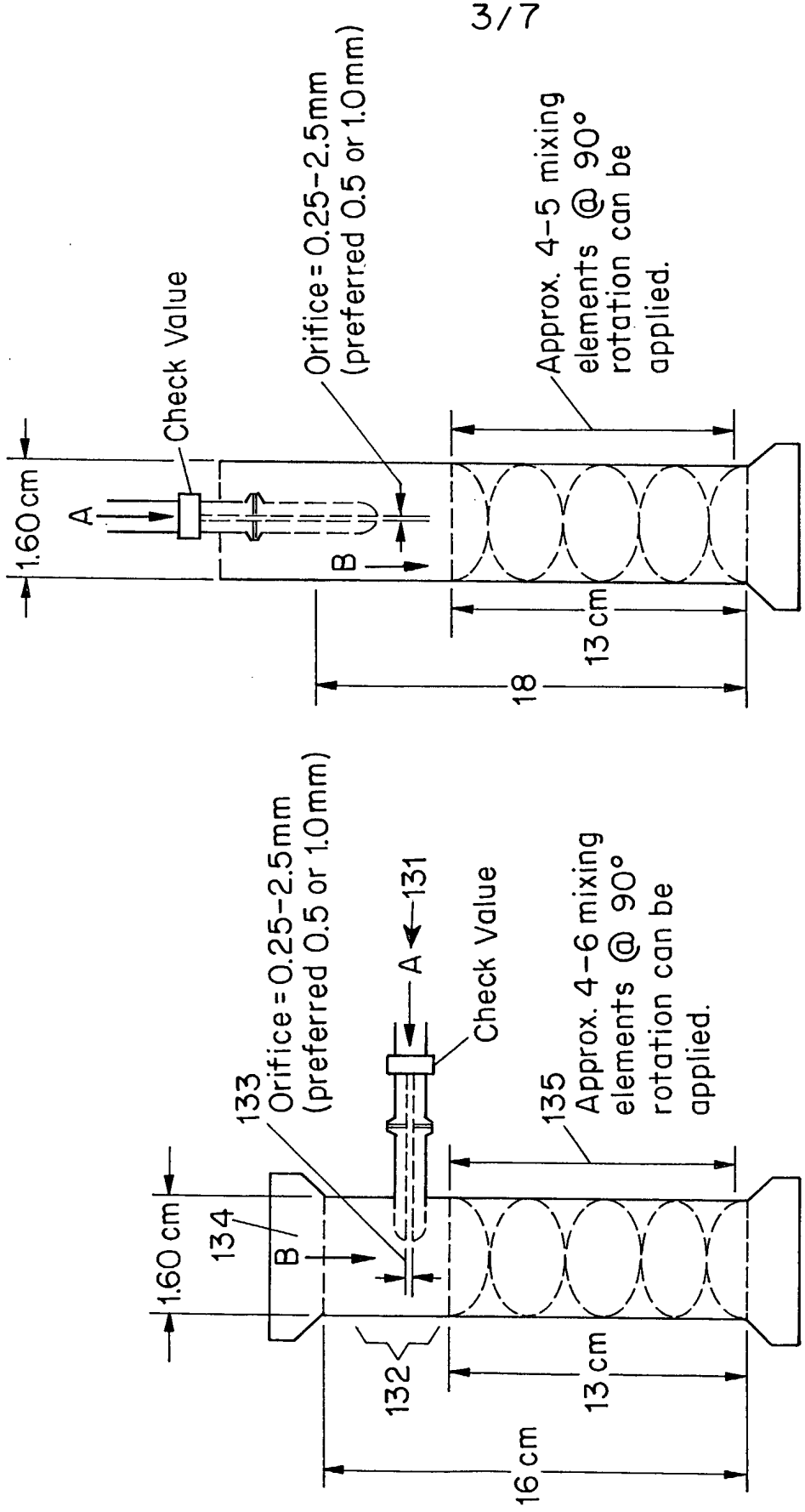


FIG. 2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13 cm
18
1.60 cm
134
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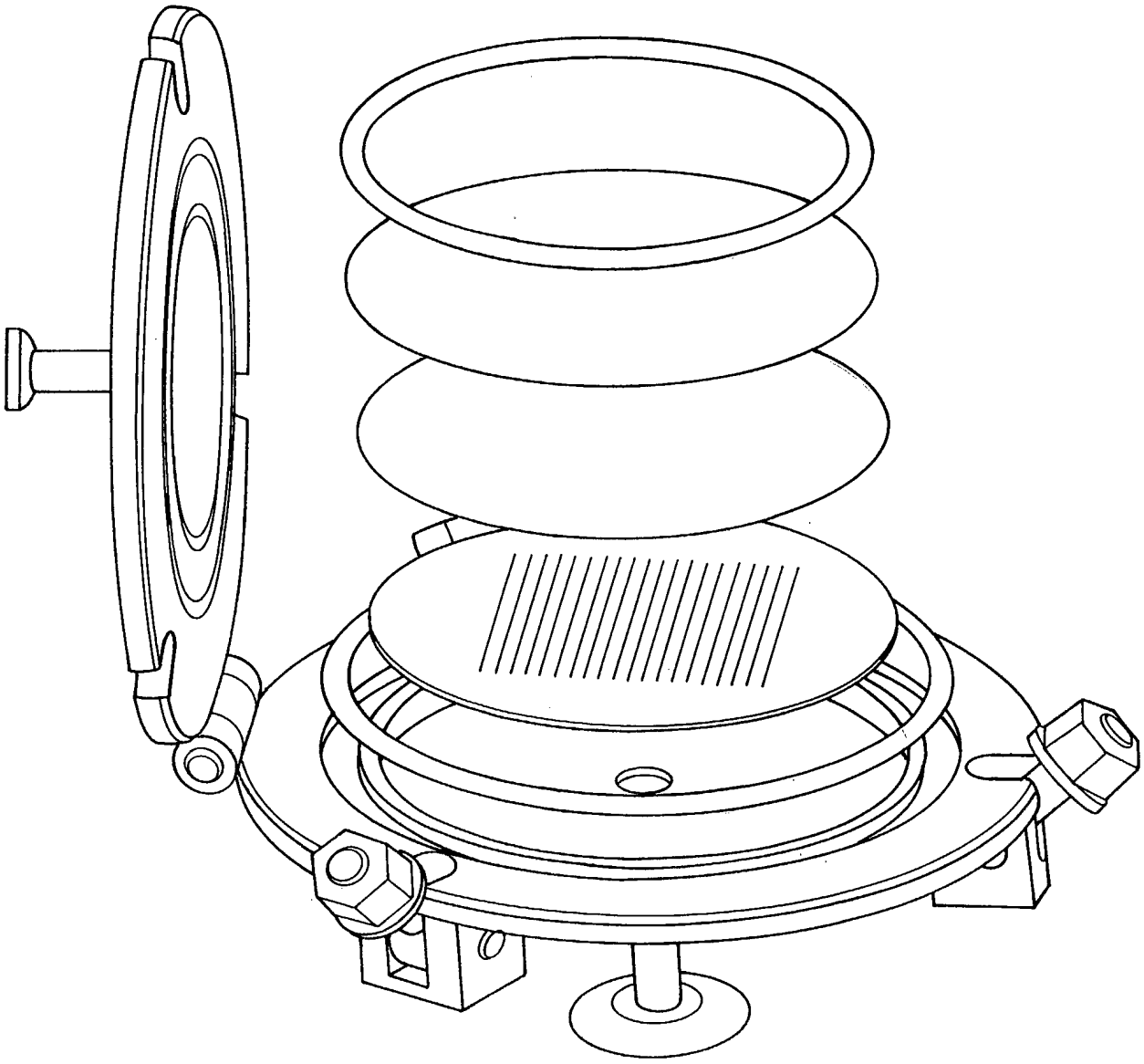
FIG. 3



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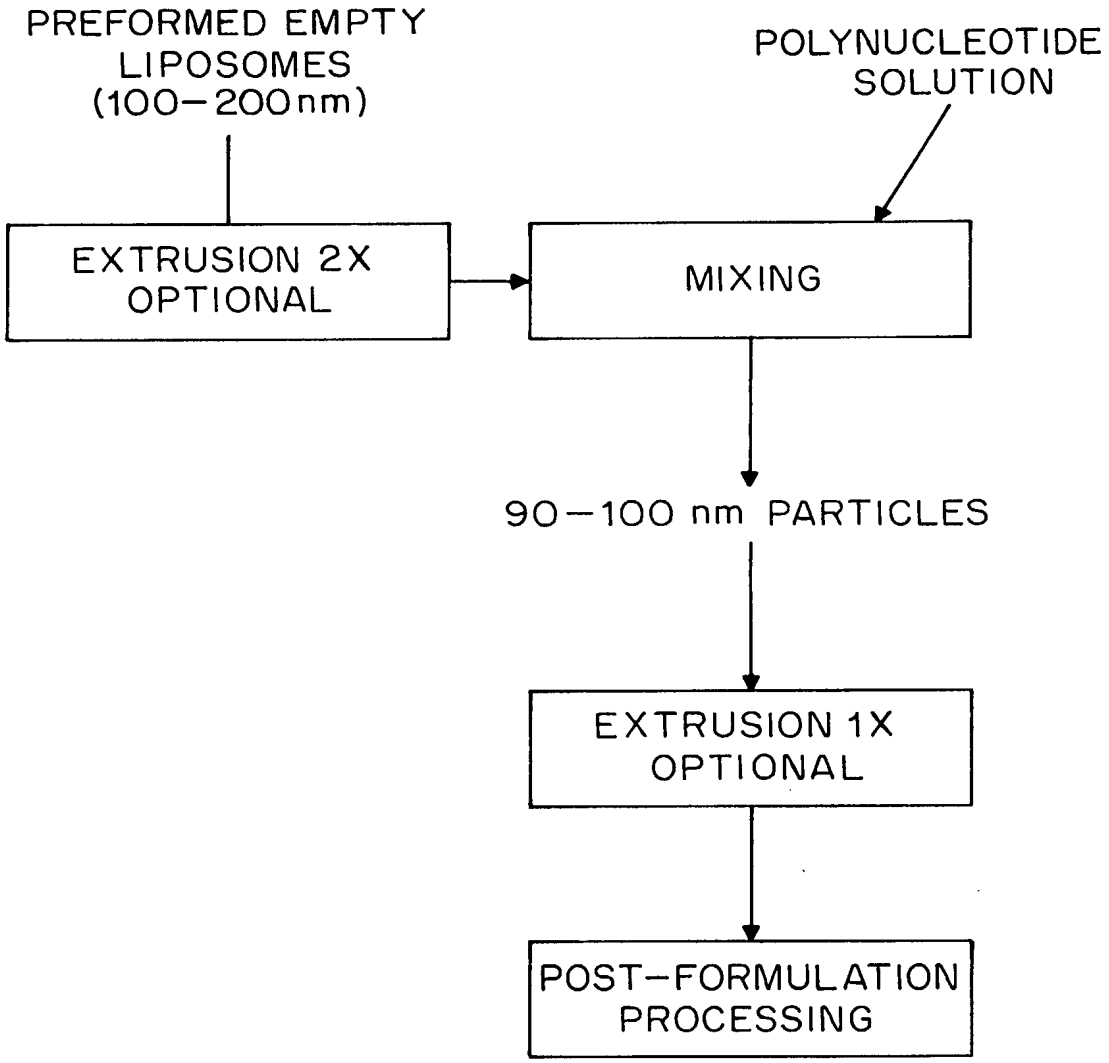
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POST–FORMULATION PROCESSING

FIG. 5

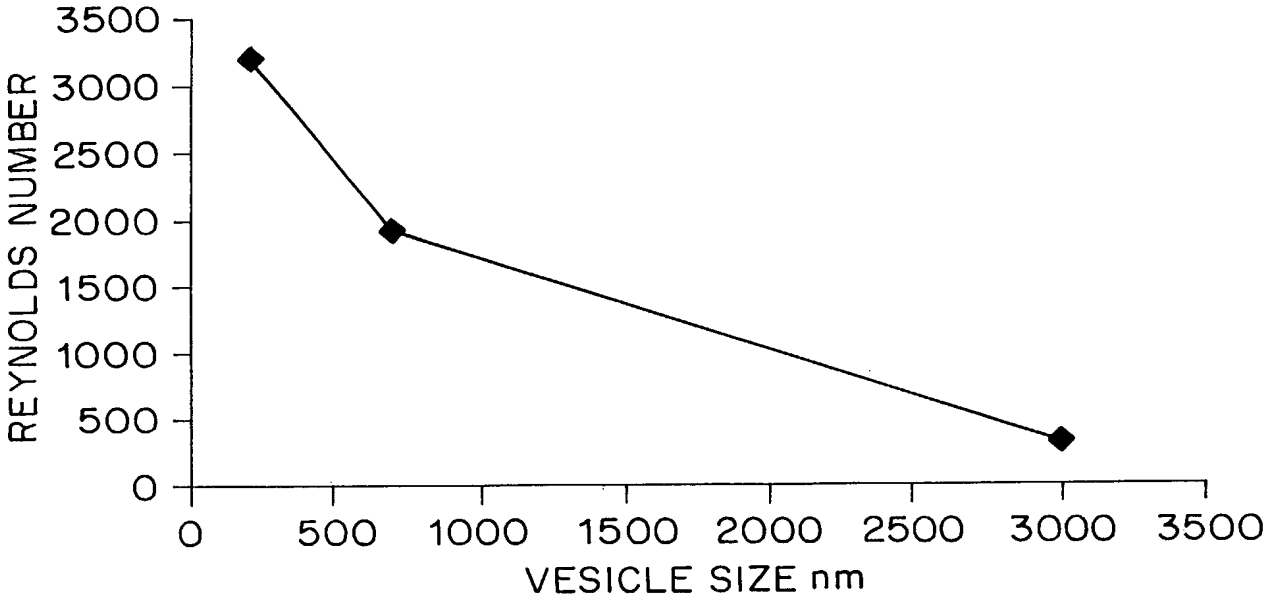
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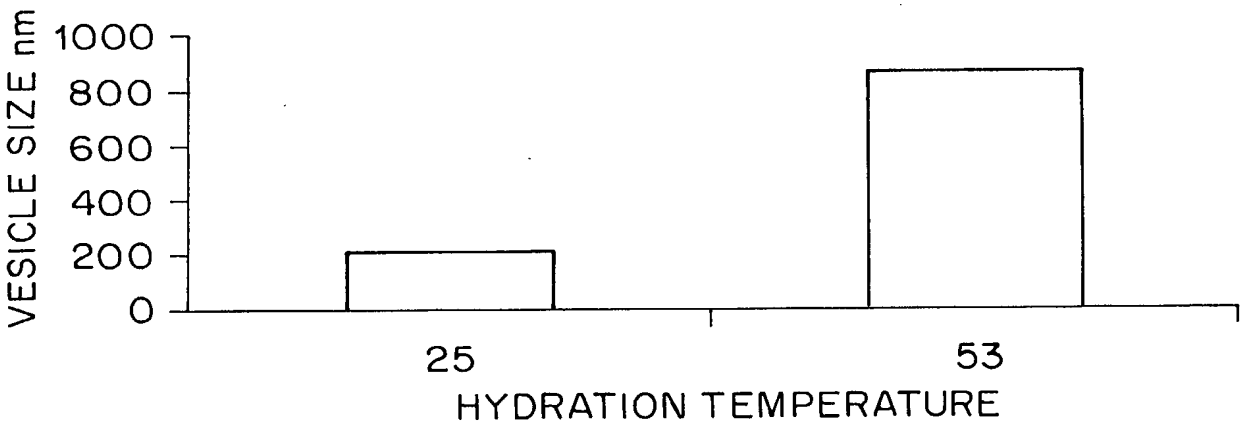
FIG. 6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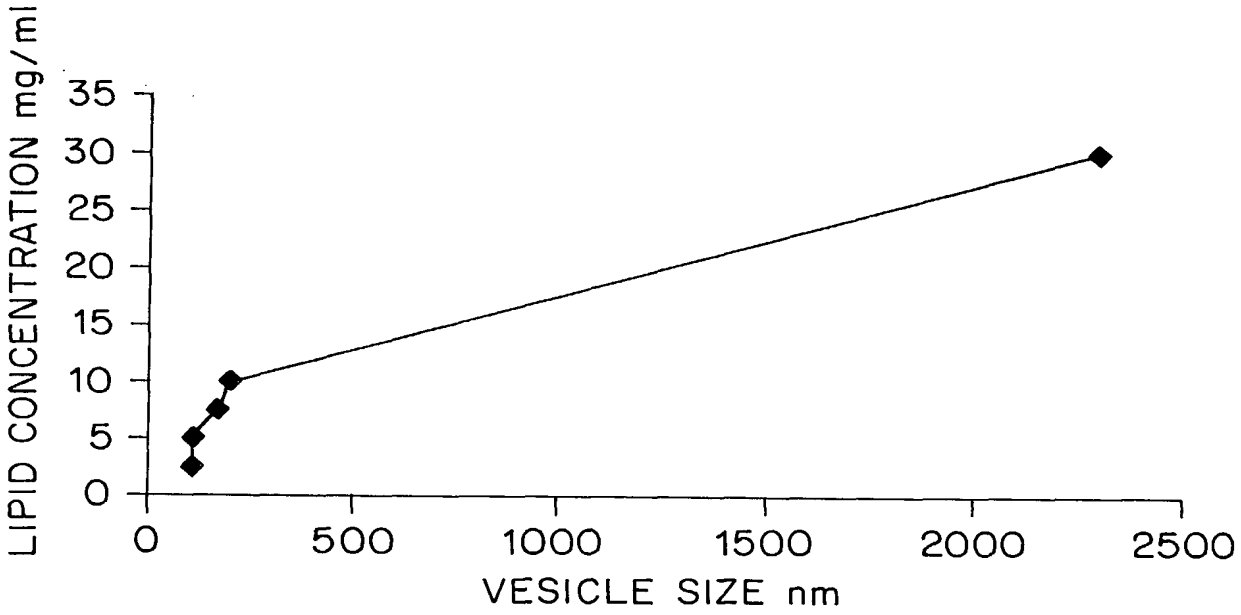
HYDRATION TEMPERATURE

FIG. 7


LIPID CONCENTRATION mg/ml
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15
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5
0
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2000
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VESICLE SIZE nm

FIG. 8

SEQUENCE LISTING

<110> Inex Pharmaceuticals Corp.
Knopov, Victor
Dzubanov, Kirill
Harper, Kevin
Cullis, Pieter R.

<120> METHODS AND APPARATUS FOR PREPARATION OF LIPID VESICLES

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Inter[redacted]onal Application No
PCT/CA 00/00842

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K9/127

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, CHEM ABS Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 25319 A (DEPOTECH CORP) 27 May 1999 (1999-05-27) page 1, line 8 - line 11 page 4, line 21 -page 5, line 19 page 8, line 4 - line 8 page 17, line 14 - line 24; figure 1 claims 1,2,7,8; example 6	1-24
A	US 5 776 486 A (CASTOR TREVOR P ET AL) 7 July 1998 (1998-07-07) column 4, line 2 - line 6 column 4, line 20 - line 25 column 5, line 45 -column 6, line 35 column 8, line 44 -column 12, line 5; figure 1 claims 14-24; examples 1,4	1-24

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 2000	24/11/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Marttin, E

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